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1 Arpin is critical for phagocytosis in macrophages and is targeted by human rhinovirus 16

2

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23

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27

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29

30

31 **Abstract**

32

33 Human rhinovirus is a causative agent of severe exacerbations of chronic obstructive  
34 pulmonary disease (COPD). COPD is characterized by an increased number of alveolar  
35 macrophages with diminished phagocytic functions, but how rhinovirus affects macrophage  
36 functions is still unknown. Here we describe that human rhinovirus 16 **impairs** bacterial  
37 uptake and receptor-mediated phagocytosis in macrophages. The stalled phagocytic cups  
38 **contain** accumulated F-actin. Interestingly, we **find** that human rhinovirus 16 down-**regulates**  
39 the expression of Arpin, a negative regulator of the Arp2/3 complex. Importantly, re-  
40 expression of the protein **rescues** the defective internalisation in human rhinovirus 16 treated  
41 cells, demonstrating that Arpin is a key factor targeted to impair phagocytosis. We further  
42 show that Arpin **is** required for efficient uptake of multiple targets, for F-actin cup formation  
43 and for successful phagosome completion in macrophages. Interestingly, Arpin **is** recruited at  
44 sites of membrane extension and phagosome closure. Thus, we identify Arpin as a central  
45 actin regulator during phagocytosis. We reveal that it is targeted by human rhinovirus 16,  
46 allowing the virus to perturb bacterial internalisation and phagocytosis in macrophages.

47

48

## 49 **Introduction**

50 Human rhinovirus (HRV) belongs to the *Picornaviradae* family. It is a small, non-enveloped  
51 virus with a single stranded, positive sense RNA genome encased within an icosahedral  
52 protein capsid with 60 copies each of four key viral proteins, VP1-VP4 [1]. Dependent on the  
53 clades the viruses use either the low-density lipoprotein receptor (LDLR) family, the  
54 intracellular adhesion molecular 1 (ICAM1) or cadherin related family member 3 to bind and  
55 enter cells [2-5]. HRV is known to productively infect epithelial cells [6-12], but the response  
56 in macrophages has received limited attention [7, 13]. Reports suggest that HRV can infect  
57 monocytes/macrophages [7, 14, 15]. A recent study demonstrated that epithelial cells directly  
58 enhance the replication of rhinovirus in monocytes [15]. Traditionally HRV is seen as an  
59 upper respiratory tract pathogen [16]. However, mounting evidence shows that HRV can  
60 infect the lower respiratory tract in patients with chronic inflammatory diseases including  
61 chronic obstructive pulmonary disease (COPD) driving disease exacerbations [17-20].  
62 Interestingly, in subjects experimentally infected with RV16, RV was ingested by recruited  
63 CD68-positive and CD11b-positive macrophages in asthmatic humans, providing direct  
64 evidence that tissue macrophages in the lower airways contribute to anti-RV responses *in vivo*  
65 [21]. How HRV disrupts macrophage/monocyte functions remains unknown, but HRV was  
66 reported to induce a defective secondary response in macrophages [13, 22-24].

67

68 An important arm of the innate immune responses is the phagocytic uptake by myeloid cells.  
69 Phagocytosis is a mechanism of internalization of large particulate material, cell debris and  
70 microorganisms [25-27]. It is strictly dependent on actin polymerization that represents the  
71 major force driving plasma membrane deformation and engulfment. Actin polymerization is  
72 induced by surface phagocytic receptors after ligation of the target and intracellular [signal](#)  
73 transduction. Phagocytic receptors include receptors for host serum factors (opsonins) such as  
74 immunoglobulin (Ig) and the complement fragment C3bi that engage Fc receptor (FcRs) and  
75 complement receptors (CR3,  $\alpha$ M $\beta$ 2), respectively, and non-opsonic receptors such as the  
76 Toll-like receptors (TLRs), the lectins and scavenger receptors [27-29]. Key players of the  
77 signaling to actin polymerization are the small GTPases of the Rho family [30, 31]. In the  
78 well-characterized FcR-mediated phagocytosis, Cdc42 activation in the nascent phagocytic  
79 cup activates effectors like N-WASP, an actin nucleation-promoting factor (NPF) that acts on  
80 the actin related protein 2/3 (Arp2/3) actin nucleation complex. Rac1 is then essential for F-  
81 actin polymerization to complete extension and closure, through activation of another NPF,  
82 the WAVE complex [26, 32, 33].

83

84 During phagosome formation, actin polymerization is transient and forms a specific F-actin  
85 ring-like structure, called the phagocytic cup. The actin ring diameter progressively shrinks  
86 until the membrane extensions eventually fuse, a step promoted by dynamin [26, 34]. Actin  
87 filaments experience a high turnover, with intense polymerization in the tips of the membrane  
88 folds and depolymerization at the base of the phagocytic cup [33, 35-38]. Several protein  
89 activities have been reported to play a role in actin remodeling, including cofilin and  
90 enzymatic activities leading to PIP(4,5)<sub>2</sub> hydrolysis or consumption [36, 38-42].

91

92 In addition, proteins inhibiting directly Arp2/3 have been described, namely Gadkin, PICK1  
93 and Arpin [43-45], for which no role in phagocytosis has been reported yet. These inhibitors  
94 are not found freely in the cytosol but localise to specific membranes like NPFs making them  
95 ideal candidates to counteract NPF activity [46]. Arpin was found to bind to the Arp2/3  
96 complex without activating it [45]. Instead Arpin exposes its COOH terminal acidic tail to  
97 inhibit the Arp2/3 complex [47, 48]. In cells studied so far, Arpin localizes at lamellipodial  
98 edges along with the WAVE complex [45]. The ability of Arpin to interact with Arp2/3 was  
99 found to depend on Rac1 signalling [45]. In response to Rac1 signalling, Arpin inhibited  
100 Arp2/3 at lamellipodial tips where Rac1 also stimulates actin polymerisation through WAVE  
101 [45]. This placed Arpin downstream of Rac1 in a cycle with Rac inducing and inhibiting actin  
102 polymerisation [45]. The major function of Arpin described to date is the inhibition of cell  
103 migration [49] and a control of cell steering [45].

104

105 In this study, we demonstrate that HRV16 impairs macrophage phagocytosis of multiple  
106 targets. We report that HRV16 induced a down-regulation of Arpin in macrophages. By re-  
107 expressing Arpin in a model cellular system where HRV16 exposure led to decreased  
108 internalisation, we could rescue this defect. Further analysis revealed that Arpin is required  
109 for efficient internalisation. Thus, Arpin plays a critical role in coordinating and orchestrating  
110 actin remodelling around internalised particles, necessary for efficient phagocytosis.  
111 Therefore, we add phagocytosis to the growing list of functions being attributed to Arpin and  
112 highlight a host cell factor specifically targeted by rhinovirus.

113

## 114 **Results**

115

### 116 **Human rhinovirus 16 impairs bacterial internalisation in human macrophages**

117 We used HRV16 for our studies because it is commonly isolated in COPD [19, 20]. We first  
118 set out to determine if macrophages could internalise bacteria after HRV16 challenge. We  
119 challenged human monocyte-derived macrophages (hMDMs) with HRV16, HRV16  
120 inactivated by a UV treatment (HRV16<sup>UV</sup>) or mock infected medium (MI) for 1 h at room  
121 temperature followed by overnight rest. The next day, we exposed them to non-typeable  
122 *Haemophilus influenzae* (NTHi), *Moraxella catarrhalis*, *Staphylococcus aureus* or  
123 *Pseudomonas aeruginosa* that are frequently associated with exacerbations of COPD [20].  
124 We measured internalisation at 30 and 120 min after washing and incubation with antibiotics  
125 to kill extracellular bacteria. We found that hMDMs challenged with HRV16 were  
126 significantly impaired in their ability to internalise all four bacteria over 120 min compared to  
127 hMDMs challenged with HRV16<sup>UV</sup> or mock infected (Figure 1A-D). To determine if this  
128 result was due to a high HRV16 tissue culture infective dose 50 (TCID<sub>50</sub>), we repeated our  
129 experiments over a range of TCID<sub>50</sub>'s and measured internalisation of NTHi and *S. aureus*  
130 over 120 min. We found that HRV16 impaired the internalisation of NTHi (Figure EV1A-E)  
131 and *S. aureus* in hMDMs (Figure EV2A-E) from TCID<sub>50</sub>'s as low as 1 x 10<sup>3</sup> reaching a  
132 maximal impairment at TCID<sub>50</sub> 1 x 10<sup>7</sup>. We also calculated the percentage inhibition of  
133 internalisation of NTHi or *S. aureus* at 120 min for all TCID<sub>50</sub>'s relative to mock infection  
134 and found that on average the impairment in internalisation caused by HRV16 was greater for  
135 *S. aureus* than NTHi at TCID<sub>50</sub> 1 x 10<sup>5</sup> or 1 x 10<sup>6</sup> but identical and maximal by TCID<sub>50</sub> 1 x  
136 10<sup>7</sup> reaching 80% (Figure EV1F and EV2F).

137  
138 Then, because reduced internalization of bacteria could be due to a reduced initial binding to  
139 the cell surface, we repeated our experiments over 30 min by incubating hMDMs with the  
140 different bacteria on ice for 5, 15 or 30 min (Figure 1E-H). No differences in bacterial  
141 attachment to hMDMs were observed in cells challenged with HRV16 or control conditions.  
142 These results indicated that HRV16 impaired the response of hMDMs to bacteria at the level  
143 of internalisation.

144  
145 Finally, because HRV16 is a predominant cause of exacerbation in COPD (Caramori *et al.*,  
146 2003, Wilkinson *et al.*, 2017), we decided to explore the effect of HRV16 challenge on  
147 bacterial internalisation by human alveolar macrophages (AMs) (table 1). We found that non-  
148 smoker, non-COPD AMs challenged with HRV16 were significantly impaired in their ability  
149 to internalise NTHi over 120 min compared to AMs challenged with HRV16<sup>UV</sup> or mock  
150 infection (MI) (Figure 1I). When we repeated our experiments using healthy smoker AMs we

151 first found that they internalised less bacteria at baseline relative to non-smoker, non-COPD  
152 AMs (Figure 1J). Interestingly, even if smoking itself impaired bacterial internalisation,  
153 HRV16 challenge led to an additive impairment in NTHi internalisation by healthy smoker  
154 AMs (Figure 1J).

155

156 Taken together, these results demonstrate that HRV16 challenge in *in vitro* derived  
157 macrophages impaired their ability to internalise bacteria. Importantly, we observed the same  
158 defect in human lung macrophages.

159

### 160 **Human rhinovirus 16 impairs zymosan, CR3- and FcR-mediated internalisation in** 161 **human macrophages**

162 We next wanted to determine if the impairment in internalisation caused by HRV16 was  
163 specific to bacteria. Therefore, we exposed hMDMs post HRV16 challenge to zymosan for 60  
164 min. We found that hMDMs challenged with HRV16 were significantly impaired in their  
165 ability to internalise zymosan compared to MI or HRV16<sup>UV</sup> treated hMDMs (Figure 2A),  
166 internalising on average 70% less zymosan (Figure 2A). To allow us to better characterize the  
167 defect in internalisation caused by HRV16 we decided to next analyse the internalisation of  
168 opsonized particles that trigger only one type of phagocytic receptor. Therefore, post HRV16  
169 challenge we exposed hMDMs to sheep red blood cells (SRBC) opsonized with either IgM-  
170 iC3b or IgG for 60 min. hMDMs challenged with HRV16 were significantly impaired in their  
171 ability to internalise either IgM-iC3b or IgG opsonized SRBC compared to control or  
172 HRV16<sup>UV</sup> treated hMDMs (Figure 2B-E), internalising on average 50% less of either particle  
173 (Figure 2B-C and EV4A showing the internalisation index). Representative images of mock  
174 infected or HRV16 treated hMDMs are shown in figure 2D-E, highlighting the internalized  
175 SRBC in green. The images clearly demonstrate the impaired internalisation caused by  
176 HRV16 towards IgM-iC3b opsonized SRBC (Figure 2D) or IgG opsonized SRBC (Figure  
177 2E).

178

179 These results along with the results in Figure 1 demonstrate that HRV16 challenge of hMDMs  
180 impairs various phagocytic pathways, suggesting that HRV16 targets a global regulator of  
181 phagocytosis in macrophages.

182

### 183 **Human rhinovirus 16 impairs phagocytic cup formation in human macrophages**

184 Having shown that HRV16 impaired internalisation by hMDMs, we next set out to analyse if  
185 HRV16 altered membrane remodelling at the onset of phagocytosis. For this, we challenged  
186 hMDMs with HRV16 or MI control and then exposed them to IgG-opsonised SRBC for 2-30  
187 min. The samples were stained with phalloidin to analyse F-actin recruitment around the  
188 internalised SRBC (Figure 3A, Z projections of stack images). We counted the number of F-  
189 actin positive cups (Figure 3B) and calculated the enrichment of F-actin around the SRBC at  
190 each phagocytic cup relative to the cell cortex (Braun 2007) (Figure 3C). Over the first 5 min  
191 of internalisation, we did not observe any significant difference in the number of F-actin cups  
192 that formed in mock infected or HRV16 treated hMDMs (Figure 3B). Despite this, there was  
193 less internalisation in HRV16 challenged hMDMs over the first 5 min compared to control  
194 hMDMs (Figure 3D). Importantly, there was no equivalent difference in SRBC association to  
195 HRV16 challenged hMDMs over these first 5 min (Figure 3E). Despite the similarity in the  
196 number of F-actin cups that formed at 5 min in both conditions, we could observe differences  
197 in the F-actin make up around the internalised SRBC. The cups looked thinner and less  
198 defined in HRV16 challenged hMDMs compared to control hMDMs, and the intensity of F-  
199 actin staining around the cups was lower in the early time point (2 min, Figure 3C). At 15 and  
200 30 min post-internalisation, we observed a sustained number of F-actin cups in HRV16  
201 challenged hMDMs compared to mock infected hMDMs where only few F-actin cups were  
202 still observed (Figure 3B) and this reached statistical significance at 15 min (Figure 3B). In  
203 line with these results, we observed significantly less internalisation over these later time  
204 points in HRV16 challenged hMDMs (Figure 3D), with no differences in association (Figure  
205 3E). Importantly, internalisation was still inhibited at 60 min in HRV16 challenged hMDMs,  
206 demonstrating that HRV16 does not just delay, but inhibits, internalisation (Figure EV4A).  
207 When we next looked at F-actin cups at 15 min, we could see they were stalled and still  
208 contained F-actin, similar to MI at 5 min, but the intensity of staining was lower (Figure 3A  
209 and C). Very interestingly, as opposed to the control cells where the cups had disassembled  
210 and only adhesion structures were detected in the images projected in Z, we observed a  
211 sustained enrichment of F-actin around internalised SRBC that was similar from 5-30 minutes  
212 and showed no clear decrease (Figure 3A,E).

213 Finally, to better visualise the membrane architecture in HRV16 challenged hMDMs, we  
214 performed scanning electron microscopy on mock infected or HRV16 challenged hMDMs  
215 after 5 min of internalisation of IgG-opsonised SRBC (Figure 3F). In mock infected hMDMs,  
216 we often observed a lot of membrane folds in several layers progressing over the SRBC  
217 (Figure 3F). In HRV16 challenged hMDMs, there were fewer membrane folds, the membrane



218 covering the SRBC was thinner and only partially covered of the SRBC (Figure 3F).  
219 Combined with our fluorescence images, these results demonstrate that HRV16 impairs  
220 internalisation in hMDMs by perturbing early phagosome formation and actin remodelling.

221

### 222 **Human rhinovirus does not affect the expression of F-actin in human macrophages but** 223 **down-regulates the expression of Arpin**

224 We next set out to determine more precisely whether HRV16 disrupted the actin cytoskeleton  
225 in hMDMs. We challenged hMDMs with HRV16 or control medium for 1 h followed by  
226 overnight rest and then stained the cells with phalloidin. The F-actin network did not appear  
227 to be disrupted by HRV16, but the global F-actin intensity was lower in HRV16 challenged  
228 hMDMs compared to mock infected hMDMs (Figure 4A). When we quantified our images  
229 we first determined that the total F-actin intensity in each field imaged was significantly lower  
230 in HRV16 challenged hMDMs compared to control hMDMs (Figure 4B). In addition, we  
231 observed that the intensity of the punctate F-actin was significantly lower in HRV16  
232 challenged *vs* mock treated hMDMs (Figure 4C). Because the primary cells are  
233 heterogeneous, we also analysed the data in figure 4C on a per cell basis and determined that  
234 the intensity of staining of punctate F-actin was significantly lower in individual cells in  
235 HRV16 challenged hMDMs compared to control conditions (Figure 4D). Collectively, these  
236 results suggested that HRV16 disrupts the F-actin network in hMDMs.

237 We therefore postulated that there might be globally less actin protein in our HRV16  
238 challenged hMDMs. However, immunoblot analysis revealed no significant change in the  
239 actin expression between HRV16 challenged (lane 3), HRV16<sup>UV</sup> challenged (lane 2) or mock  
240 infected cells (lane 1) (Figure 4E-F). Although the quantification across 10 donors revealed  
241 variations in the global amount there was no significant trend in either direction towards more  
242 or less actin in either condition (Figure 4F). This result suggested that HRV16 might be  
243 targeting a regulator of the F-actin network in hMDMs rather than the G-actin pool. To test  
244 this hypothesis, we challenged hMDMs to HRV16 or HRV16<sup>UV</sup> or mock conditions and  
245 screened for a range of actin-associated proteins. Of the actin regulators that we tested, we  
246 found that the Arp2/3 inhibitor Arpin was consistently and significantly down-regulated on  
247 average by 30% in HRV16 challenged hMDMs compared to control conditions (Figure 4E-  
248 G). Of note, there was no effect on the global expression of Rac1, which is upstream of Arpin  
249 and activates Arp2/3 via WAVE (Eden 2002) (Figure 4G). We found no significant decrease  
250 in the expression of Cdc42 that activates Arp2/3 via WASP (Figure 4G). We then looked at  
251 subunits of Arp2/3 including ArpC2 (p34-Arc) and ArpC5 (p16-Arc). We observed no

252 difference in the global expression of p34-Arc in HRV16 challenged hMDMs compared to  
253 control conditions and a non-significant increase in the global expression of p16-Arc that was  
254 not specific to the live virus (Figure 4G). We also analysed the expression of cofilin and  
255 observed no difference in the total or phosphorylated forms (Figure 4H-I, ratio).

256

257 Finally, to further confirm that the disruption of Arpin expression by HRV16 was mediating  
258 deficient internalisation caused by HRV16, we set out to re-express the protein and analyse if  
259 internalisation is restored. To overcome the issues of transfecting primary human  
260 macrophages, we made use of retinal pigment epithelial-1 (RPE-1) cells that were transduced  
261 with lentivirus to express the phagocytic FcγRIIA/CD32. We first verified by flow cytometry  
262 that the transduced cells expressed the receptor and found that 100% of cells were positive for  
263 CD32 (Figure EV5A). The RPE-1-FcγRIIA cells were able to specifically phagocytose IgG-  
264 opsonized SRBC as compared with non-opsonized particles, while the parental cells were not  
265 competent for this phagocytosis (Figure EV5B-D). Next, we exposed these cells to HRV16 or  
266 to mock infected medium for 1 h followed by overnight rest and then challenged them with  
267 IgG-opsonised SRBC for 1 h. We found that RPE-1-FcγRIIA cells exposed to HRV16  
268 internalised on average 40% less SRBC compared to a mock treatment (Figure EV5D).  
269 Having confirmed that RPE-1-FcγRIIA cells responded like human macrophages to HRV16,  
270 we exposed these cells to HRV16 or mock medium and, after the overnight rest, transfected  
271 them with plasmids encoding either EGFP or EGFP-Arpin for 24 h (Figure 4J,K). Then we  
272 performed a phagocytosis experiment using IgG-opsonised SRBC for 1 h. We demonstrated  
273 that RPE-1-FcγRIIA cells exposed to HRV16 and then transfected with EGFP internalised  
274 significantly fewer SRBC compared to mock infection controls, which averaged an  
275 approximate 40% decrease (Figure 4K). Importantly, when RPE-1-FcγRIIA cells were  
276 exposed to HRV16 and then transfected to express EGFP-Arpin, the efficiency of  
277 internalisation of SRBC was restored (Figure 4K).

278

279 Taken together, our results show that live HRV16 impairs the F-actin network in hMDMs and  
280 reveal that the Arp2/3 inhibitor Arpin is downregulated in live HRV16-treated macrophages.  
281 Furthermore, these results demonstrate that HRV16 specifically targeted Arpin to decrease  
282 internalisation and that transient expression of the protein restored the phagocytic capacities  
283 of HRV16-treated cells. Therefore, Arpin is a crucial factor targeted by this rhinovirus to  
284 impair phagocytosis.

285

## 286 **Arpin knockdown impairs bacterial internalisation**

287 Because the Arp2/3 inhibitor had never been implicated in phagocytosis, we next addressed  
288 whether a decrease in Arpin expression could recapitulate the perturbations in phagosome  
289 formation that we observed in macrophages challenged with HRV. We treated hMDMs with  
290 two siRNA against Arpin [45] or luciferase as a control, or left them non-treated for 96 h.  
291 Immunoblot analysis and quantification demonstrated significant Arpin reduction for both  
292 siRNA sequences in 9 donors (Figure 5A and 5B). We then challenged the Arpin-depleted  
293 macrophages with either NTHi, *M. catarrhalis*, *S. aureus* or *P. aeruginosa* for up to 120 min.  
294 We found that hMDMs treated with Arpin siRNA were significantly impaired in their ability  
295 to internalise all bacteria (Figure 5C-F) relative to non-treated hMDMs. Importantly, hMDMs  
296 treated with siLuciferase demonstrated similar bacterial internalisation to non-treated hMDMs  
297 (Figure 5C-F). Finally, we also assessed bacterial binding to hMDMs over 5-30 min of  
298 infection post siRNA treatment and found no difference between the four conditions (Figure  
299 EV3A-D). These results demonstrate that Arpin plays a crucial role in bacterial internalisation  
300 in hMDMs.

301

## 302 **Arpin knockdown impairs membrane extension around internalised particles**

303 Having shown that Arpin knockdown impaired internalisation of bacteria by human  
304 macrophages, we next set out to analyse if it impaired F-actin dynamics at the phagocytic cup.  
305 We treated hMDMs with Arpin siRNA or controls and after 96 h challenged them with IgG-  
306 opsonised SRBC for 15-30 min (Figure 6A-D). Significantly fewer SRBC were internalized  
307 in macrophages when Arpin was knocked down, as compared with control conditions (Figure  
308 6B). In contrast, there was a non-significant trend towards less association of SRBC to  
309 hMDMs treated with siRNA against Arpin (Figure 6C), indicating that depletion of Arpin  
310 leads to a defective phagocytosis. Similar to what we observed with HRV16, internalisation  
311 was not simply delayed in Arpin-depleted macrophages, as internalisation was still impaired  
312 after 60 min (Figure EV4B). In addition, we observed significantly more F-actin cups in  
313 Arpin-depleted macrophages as compared to control conditions at 15 min post internalisation  
314 (Figure 6 A and D). We then examined the F-actin cups in Arpin-depleted *versus* non-treated  
315 or siLuciferase treated hMDMs where the F-actin cups were complete, with homogeneous F-  
316 actin organisation around the particles (Figure 6A). In contrast, Arpin knockdown not only  
317 led to an increased number of cups visible in figure 6A, but also to cups that appeared  
318 fragmented with puncta of staining (Figure 6A). By 30 min post-internalisation there were  
319 very few cups in control conditions (Figure 6A, D). However, in Arpin knockdown hMDMs,

320 there were still significantly more F-actin cups compared to control conditions albeit the  
321 global number had decreased (Figure 6D). We next calculated the enrichment of F-actin  
322 around internalised SRBC relative to the cell cortex. As expected, the enrichment of F-actin  
323 around internalised SRBC was similar for non-treated or siLuciferase treated hMDMs at 15  
324 min and decreased by 30 min (Figure 6E). Strikingly, in Arpin-depleted hMDMs, we  
325 observed significantly more F-actin enrichment around internalised SRBC at 15 and 30 min  
326 relative to control conditions (Figure 6E), consistent with the increased number of F-actin  
327 cups counted (Figure 6D). To better observe the membrane architecture when Arpin was  
328 knocked down in human macrophages, we performed scanning electron microscopy on Arpin  
329 siRNA or control siRNA-treated hMDMs after 15 min of internalisation of IgG-opsonised  
330 SRBC (Figure 6F). In control hMDMs, we could see a very thin covering of host membrane  
331 over the SRBC indicating near-complete internalisation (Figure 6F). In hMDMs treated with  
332 siRNA targeting Arpin, we could observe accumulation of membrane folds around the SRBC  
333 or thin membrane layers partially covering the particle (Figure 6F), which was reminiscent of  
334 the phenotype obtained after HRV16 treatment. This indicated that Arpin knockdown was  
335 impairing phagocytic cups progression and completion.

336

337 To further describe the role of Arpin in phagosome formation, we used the RAW264.7 murine  
338 cells transfected to transiently express lifeact-mCherry to detect polymerized branched actin  
339 and EGFP-Arpin. We performed the 3D phagosome formation and closure assay that we set  
340 up using Total Internal Reflection Fluorescence Microscopy (TIRFM) [34, 36, 50, 51] (Figure  
341 6G-H). In this assay, living cells phagocytose particles that are non-covalently attached to the  
342 coverslips, allowing for observation of the base of the phagocytic cup in the epifluorescence  
343 mode when the stage is moved 3  $\mu\text{m}$  in z, the extending pseudopods, as well as the precise  
344 site of phagosome scission in the TIRFM mode (Figure 6G). We observed that Arpin was  
345 recruited at the site of phagosome formation with branched actin in the extending membrane  
346 folds (Figure 6H). The epifluorescence images showed that Arpin was not accumulated at the  
347 base of the phagosome in the region where actin is cleared. Interestingly, Arpin was enriched  
348 at the site of phagosome closure detected in both epifluorescence and TIRFM modes. Arpin is  
349 thus potentially playing a very local role in the actin polymerization/depolymerization cycle,  
350 rather than a role in the actin clearance at the base of the phagocytic cup.

351

352 Together, these results show that Arpin is not required for the initial onset of actin  
353 polymerization but regulates the local branched actin network for a successful phagocytic cup  
354 extension and closure.

355

356

357 **Discussion**

358

359 In this study, we show that HRV16 impairs the internalisation of bacteria and other particles  
360 by human macrophages. We reveal that Arpin is a host cell protein downregulated by the  
361 virus that is necessary and sufficient for the internalisation defect driven by HRV16 as re-  
362 expression rescued deficient internalisation in RPE-1 cells exposed to the virus. We further  
363 demonstrate for the first time that this Arp2/3 inhibitor is critical for efficient receptor-  
364 mediated phagocytosis and bacterial uptake in macrophages.

365

366 Our understanding of how macrophages, including differentiated tissue macrophages, deal  
367 with HRV infections compared to epithelial cells is incomplete [52]. There are reports  
368 indicating that HRV can infect monocytes/macrophages *in vitro* and *in vivo* [7, 14, 15, 20, 21,  
369 23]. In our work, we studied how differentiated human macrophages and resident lung  
370 derived macrophages are affected by HRV infection. We first found that post HRV16  
371 exposure, macrophages did not internalise a range of gram-positive and gram-negative  
372 bacteria that are frequently associated with exacerbations [20]. Interestingly, we observed an  
373 innate impairment of bacterial internalisation in alveolar macrophages from healthy smokers  
374 compared to non-smokers controls that was further exacerbated following exposure to  
375 HRV16. The ability of smoking to worsen viral infections has been suggested, but the reports  
376 are still conflicting (for a review, see [53]). Because reduced phagocytic capacities could be  
377 due to reduced surface phagocytic receptor expression [54], we explored whether bacterial  
378 binding to macrophages was affected. We found that this was not the case, further  
379 emphasising that HRV16 specifically affected the internalisation step. These results are  
380 important in the context of COPD where HRV is frequently isolated [20] and, together with  
381 other defective functions like cell activation [13, 22-24], could be one of the explanations as  
382 to why patients show bacterial outgrowths post HRV infection [13, 19].

383

384 Phagocytosis is strictly dependent on actin polymerization (for review, [26, 55], and upon  
385 phagocytosis, actin polymerization at the site of particle binding is transient and intense and  
386 corresponds to the formation of a phagocytic cup. We demonstrate here that, although HRV16  
387 challenged macrophages were impaired in internalisation over 30 min, they could form F-  
388 actin cups. However, there was an inherent delay to complete phagocytosis and to clear F-  
389 actin from forming phagosomes. Importantly, we were able to demonstrate that, while in  
390 mock infected macrophages actin disassembled after 30 min, there was a maintenance of F-

391 actin in phagocytic cups in HRV16 challenged macrophages. When we analysed the  
392 membrane extension over particles by scanning electron microscopy, we first observed that in  
393 contrast to monocytes [36], hMDMs seemed to have more folds of membrane around the  
394 particles. While there were multiple ruffles and folds of membrane in uninfected macrophages  
395 at 5 min, in HRV16 challenged macrophages, the membrane was a lot thinner and only  
396 partially covered the particle. In Arpin knockdown macrophages, we observed two  
397 phenotypes, either a very thin covering of membrane over the internalised particle, similar to  
398 HRV16, or small partial folds of membrane below the particle. All these results suggested that  
399 HRV16 challenged macrophages and Arpin-depleted macrophages show similar defects in  
400 their ability to promote actin remodelling and to extend membranes for efficient phagosome  
401 completion.

402

403 Arpin is a newly discovered inhibitor of the Arp2/3 complex that counteracts the activatory  
404 signals provided by the WAVE/Rac1 complex [45]. In addition to its reported role in cell  
405 steering and in controlling the lifetime of lamellipodia [45, 49], it was shown to be  
406 dispensable for chemotactic migration of cancer cells and of *Dictyostelium discoideum* [56].  
407 Of note, we found no significant difference in the spontaneous migration of macrophages  
408 between HRV treated and control cells (Appendix Figure 1). As it has been documented that  
409 the Arp2/3 complex is critical for phagocytosis [57], and despite a recent report [58], we  
410 postulated that the ability of Arpin to deactivate the Arp2/3 complex at sites of phagosome  
411 formation would also be important for efficient phagocytosis and that Arpin could play a role  
412 in this. Indeed, siRNA knockdown of Arpin impaired the internalisation of multiple bacteria  
413 as well as opsonized particles by macrophages without affecting their association. It has to be  
414 noted that the reduction in FcR-mediated phagocytosis measured in RPE-1 cells or in  
415 macrophages upon HRV16 treatment is lower than the reduction in initial uptake of bacteria  
416 after viral challenge. Other factors might be implicated in the virus-induced defective uptake  
417 of bacteria, in addition to Arpin. We found that Arpin was localized with branched actin in the  
418 extending membrane folds in the nascent phagosome, as well as at sites of phagosome  
419 scission, and not at the base of the phagosome where F-actin is cleared and where some other  
420 enzymatic activities like PLC or OCRL, involved in the actin depolymerization, have been  
421 reported to be preferentially localized (Marion et al, 2012, Schlam et al, 2015). These results  
422 suggest that Arpin is playing a very local role in the actin polymerization/depolymerization  
423 cycle and regulation of the Arp2/3 complex, rather than a role in the actin clearance at the  
424 base of the phagocytic cup, which further expands our knowledge on the network of

425 regulatory elements required for the dynamic actin remodelling and polymerization/  
426 depolymerisation cycles that are necessary for efficient phagosome building by macrophages  
427 [26].

428

429 The Arpin protein level was decreased in HRV16-exposed human macrophages, which could  
430 be the consequence of different downregulation mechanisms. We analysed the mRNA levels  
431 of Arpin in HRV16 exposed hMDMs and there was a non-significant trend towards less  
432 expression also (Appendix Figure 2), indicating that HRV16 could affect Arpin expression at  
433 mRNA level in hMDMs. The virus could regulate transcription factors binding in the  
434 promoter region of the Arpin gene like NF- $\kappa$ B RelA, c-Rel and En1, as it has been shown for  
435 different promoters in HRV16 infected epithelial cells [59-61]. In addition, there could be  
436 miRNA regulation by the virus controlling mRNA stability and/or protein translation, as well  
437 as mechanisms of protein degradation. Interestingly, interrogation of public transcriptomic  
438 dataset [62] of alveolar macrophages from COPD-smokers, healthy smokers and non-  
439 smokers, revealed many genes differentially expressed in COPD-smokers vs healthy smokers.  
440 Pathway analyses indicated a downregulation of the mechanisms of actin polymerization or  
441 cytoskeletal reorganization, which further emphasizes that actin regulation is key in COPD.

442

443 Our results demonstrate that Arpin is both necessary and sufficient for the impairment in  
444 internalisation driven by HRV16. This provides key insights into a major molecular player to  
445 explain how HRV16 is able to impair internalisation in hMDMs, which significantly builds on  
446 previous research (Oliver et al., 2008). Of note, the impairment of phagocytosis might be  
447 secondary to the establishment of viral factories hijacking cellular machineries and  
448 intracellular trafficking in the infected macrophages. In addition, the inhibition of bacterial  
449 uptake together with the inhibition of the cell inflammatory activity probably provides a  
450 beneficial environment for the virus itself.

451 In conclusion, our results demonstrate that Arpin is crucial for successful phagocytosis in  
452 macrophages and adds phagocytosis as a new function we can now attribute to this protein.

453 We reveal that Arpin is a major target of HRV16 infection, which opens new avenues for  
454 strategies to improve clearance of apoptotic cells and reduce inflammation and to increase  
455 bacterial uptake and suppress colonisation in the airways.

456

457



458 **Methods**

459

460 **Antibodies and reagents**

461 The following primary antibodies were used: mouse anti-actin (clone AC-40; Sigma, A3853),  
462 mouse anti-Cdc42 (BD Bioscience, 610929), mouse anti-Rac1 (BD Bioscience, 610650),  
463 rabbit anti-Arpin (kind gift from Alexis Gautreau), mouse anti-p16 (Synaptic Systems,  
464 305011), rabbit anti-p34 (Merck Millipore, 07-227), rabbit anti-phospho cofilin (Cell  
465 Signalling #3313), mouse anti-total cofilin (Cell Signalling, Clone D3F9, #5175), mouse anti-  
466 tubulin alpha (clone DM1A, Sigma, T9026) and purified rabbit anti-SRBCs (IGN  
467 Biochemicals). DAPI was from Sigma (D9542) and phalloidin-Cy3 from Life Technologies  
468 (A22283). Zymosan A (Sigma-Aldrich) was coupled to Cy2 (GE Healthcare). Secondary  
469 antibodies were all from Jackson Immunoresearch and were as follows: Cy5 or 488-labeled  
470 F(ab')<sub>2</sub> anti-rabbit IgG, and mouse or rabbit anti-IgG-HRP. siRNA sequences were as  
471 follows: 5'-GGAGAACUGAUCGAUGUAUCU-3' (Arpin sequence 1) and 5'-  
472 GCUUCCUCAUGUCGUCCUACA-3' (Arpin sequence 2), [45]. The control siRNA-  
473 targeting Luciferase was 5'-CGUACGCGGAAUACUUCGA-3'.

474

475 **Cell culture**

476 Human peripheral blood mononuclear cells (PBMCs) were isolated from whole blood of  
477 healthy donors (Etablissement Français du Sang Ile-de-France, Site Trinité, Inserm agreement  
478 #15/EFS/012 and #18/EFS/030 ensuring that all donors gave a written informed consent and  
479 providing anonymized samples) by density gradient sedimentation using Ficoll-Plaque (GE  
480 Healthcare). This was followed by adhesion on plastic at 37°C for 2 h and culture in the  
481 presence of adhesion medium (RPMI 1640 (Life Technologies) supplemented with 100 µg/ml  
482 streptomycin/penicillin and 2 mM L-glutamine (Invitrogen/Gibco). Then, the adhered cells  
483 were washed once with warm adhesion medium and left to rest in macrophage medium  
484 (RPMI 1640 supplemented with 10% FCS (Eurobio), 100 µg/ml streptomycin/penicillin, and  
485 2 mM L-glutamine). On day 1, the cultures were washed with adhesion medium and then  
486 supplemented every 2 days with fresh macrophage medium. The adherent monocytes were  
487 left to differentiate into macrophages as described previously [63] and used for experiments at  
488 day 10.

489

490 Human alveolar macrophages (AMs) were obtained by bronchoalveolar lavage fluid (BALF)  
491 of lung specimens from non-smokers or healthy smokers (Protocole de recherche non  
492 interventionnelle, Number ID RCB 2015-A01809) ensuring that all donors signed a consent  
493 form. The health condition of the patient was registered before the samples were treated  
494 anonymously. The sample was initially centrifuged at  $290 \times g$  for 5 minutes, the pellet  
495 resuspended in the original volume in adhesion medium and the cell count obtained. Cells  
496 were then plated onto plastic and incubated for 4 h in adhesion medium at  $37^{\circ}\text{C}$ . They were  
497 then washed thoroughly with adhesion medium and rested overnight in AM media (X-VIVO  
498 10 without phenol red and gentamicin (Lonza) supplemented with  $50 \mu\text{g/ml}$   
499 streptomycin/penicillin, 1 Mm L-glutamine and  $20 \mu\text{g/ml}$  amphotericin B (Sigma)) and  
500 experiments were performed the next day.

501

502 HeLa Ohio cells were purchased from the European Collection of Authenticated Cell Cultures  
503 (ECACC) and were cultured in DMEM GlutaMax containing 25 mM D-glucose and 1 mM  
504 sodium pyruvate (Life Technologies) supplemented with 10% FCS,  $100 \mu\text{g/ml}$   
505 penicillin/streptomycin and 2 mM L-glutamine. They were passaged every 3 days.

506

507 RAW264.7 macrophages were grown in complete medium, consisting of RPMI1640-  
508 glutamax supplemented with 10 mM HEPES, 1mM sodium pyruvate,  $50\mu\text{M}$   $\beta$ -eta-  
509 mercaptoethanol, 2 mM L-Glutamine and 10 % FCS (all from Gibco). Cells were transfected  
510 by electroporation with the Electrobuffer kit (Cell project). Routinely, one 100 mm plate of  
511 cells was grown to sub-confluence and  $10 \mu\text{g}$  of each plasmid was used for co-transfections.  
512 The cells were electroporated in 0.4 cm cuvettes (Biorad) at 250 V,  $900 \mu\text{F}$  in an  
513 electroporation apparatus (X Pulser Bio-Rad Laboratories), then immediately resuspended in  
514 complete culture medium. Efficiency of transfection was 10–40%.

515

#### 516 **Generation of RPE-1-FcγRIIA cells.**

517 FcγRIIA gene was amplified by PCR from the pRK5-FcγRIIA plasmid (E. Caron, Imperial  
518 College, London) using the following oligos: forw: 5'-  
519 CCGGCGGCCGCTCTCCAAGGTGTCC-3', and rev: 5'-  
520 GGCCCGACCGTTTATTACTGTTGACATGG-3' carrying respectively NotI and  
521 AgeI restriction site. The NotI/AgeI digested amplicons were inserted in a pLEX MCS  
522 plasmid (Open Biosystems) digested as well and dephosphorylated. Lentiviral particles were

523 produced by co-transfection of HEK293T cells with packaging plasmids (pCMV 8.91 and  
524 pEnv<sub>VSVG</sub>) and pLEX-FcγRIIA plasmid. After 48 h of culture at 37°C, the virus-containing  
525 supernatant was filtered and ultracentrifuged at 60,000 × g for 90 min at 4°C on a PEG  
526 cushion (50 %). The virion-enriched pellet was resuspended in PBS and aliquoted for storage  
527 at -80°C. hTERT RPE-1 cells (ATCC<sup>(R)</sup> n° CRL-4000<sup>TM</sup>, BIOPHENICS facility, Institut  
528 Curie, Paris) were infected with lentiviral particles from MOI 1 to MOI 10. FcγRIIA-  
529 expressing hTERT RPE-1 cells were cultured in Dubelcco's modified Eagle medium  
530 (DMEM) F-12 (Thermo Fisher Scientific) supplemented with 10 % Fetal Calf Serum (FCS,  
531 Gibco), 10 µg/ml hygromycin B and 2.5 µg/ml puromycin (Sigma). They were passaged  
532 every two days.

533

#### 534 **RPE-1 cell transfection**

535 RPE1 cells at 80 % confluence were washed with PBS and detached using 0.25 %  
536 Trypsin/EDTA (Life Technologies). After centrifugation, the cell count was determined and  
537 the cells seeded on coverslips at a density of 15, 000 per coverslip and allowed to adhere  
538 overnight. The next day, the coverslips were exposed to HRV16 or mock infected as  
539 described above. Then they were transferred to a 6 well plate and transfected. The plasmid  
540 solution was prepared in OptiMEM medium (GlutaMAX supplemented, Gibco), containing  
541 Fugene reagent (Invitrogen) and each plasmid at a concentration of 3 µg. Plasmid solution  
542 was added to each well and cultures left for 24 h at 37°C before being treated for FcR  
543 phagocytosis.

544

#### 545 **Bacterial strains and culture**

546 NTHi strain RdKW20 [64, 65] and *Moraxella catarrhalis* strain 25293 [66] were purchased  
547 from the American Type Culture Collection (ATCC). *Staphylococcus aureus* strain  
548 160201753001 and *Pseudomonas aeruginosa* strain 160601067201 from blood culture were  
549 provided by Professor Claire Poyart (Cochin Hospital). NTHi, *S. aureus* and *P. aeruginosa*  
550 were cultured on chocolate agar plates and *M. catarrhalis* was cultured on brain-heart  
551 infusion (BHI) agar plates. Plates were incubated for 24 h at 37°C until colonies appeared. All  
552 strains were grown in LB medium but for NTHi, this was also supplemented with 10 µg/ml  
553 hemin and 1 µg/ml nicotinamide adenine dinucleotide (NAD).

554

#### 555 **Human rhinovirus production**

556 Human Rhinovirus 16 (HRV16) (VR-283, strain 11757, lot 62342987) was purchased from  
557 the ATCC and stocks were produced by infecting HeLa Ohio cells in virus medium (DMEM  
558 GlutaMax containing 25 mM D-glucose and 1 mM sodium pyruvate supplemented with 10%  
559 FCS and 2 mM L-glutamine) as described previously [67]. Briefly, HeLa Ohio cells were  
560 grown to 80% confluence and infected with 5 ml HRV16 or control media for 1 h at room  
561 temperature with agitation. The remaining solution was made to 10 ml and the cells with  
562 HRV16 left for 48 h to allow for 90% CPE to develop. Supernatants were then clarified by  
563 centrifugation and filtration [67] and 1 ml stocks were produced and stored at -80°C. To UV  
564 inactivate HRV16 it was treated with UV light (1000mJ/cm<sup>2</sup>) for 20 minutes. Inactivation was  
565 confirmed by adding the inactivated virus to HeLa Ohio cells and checking for CPE.

566

#### 567 **Quantification of the tissue culture infective dose 50 (TCID<sub>50</sub>) of HRV16**

568 HeLa Ohio cells were cultivated in 96 well plates at 1 x 10<sup>5</sup> cells/well for 24 h. HRV16 was  
569 diluted 10-fold from undiluted to 10<sup>-9</sup> in virus medium 50 µl of each dilution was added to the  
570 cells in 8 replicate wells. 50 µl of virus medium was added to 2 groups of control wells in 8  
571 replicate wells per group. Cultures were incubated for 4 days at 37°C until CPE was observed  
572 in 50% of wells. TCID<sub>50</sub> was calculated using the Spearman-Kärber formula as previously  
573 outlined [67].

574

#### 575 **HRV16 and bacterial infection of human macrophages**

576

577 Macrophages were washed once in PBS and rested in virus medium. HRV16, HRV16<sup>UV</sup> or  
578 MI supernatants were added to the macrophages and placed at room temperature for 1 h with  
579 agitation to achieve a TCID<sub>50</sub> of 1 x 10<sup>7</sup>/ml. Cultures were then washed with virus medium  
580 and rested in macrophage medium overnight.

581

582 NTHi, *M. catarrhalis*, *S. aureus* or *P. aeruginosa* were grown until mid-log growth phase,  
583 centrifuged at 1692 x g for 5 min and re-suspended in 1 ml phagocytosis medium (RPMI  
584 supplemented with 2 mM L-glutamine). Bacteria was added to macrophages pre-treated with  
585 HRV16, HRV16<sup>UV</sup> or MI to achieve a multiplicity of infection (MOI) of 10/cell. Cultures  
586 were then centrifuged at 602 x g for 2 min and placed at 37°C, 5% CO<sub>2</sub> for 30 or 120 min. At

587 each time point, cultures were washed with PBS and treated with 100 µg/ml gentamicin  
588 (NTHi, *S. aureus*, *P. aeruginosa*) or 20 µg/ml (*M. catarrhalis*) for 20 min. Cultures were  
589 washed and lysed in saponin as previously described [63] and colony forming units (CFU)  
590 estimated using the Miles-Misra technique [68].

591

### 592 **Measurement of bacterial binding**

593

594 Macrophages were challenged with HRV16 or controls as described above and bacteria was  
595 prepared in the same manner. Cultures were washed with PBS and bacteria were added to  
596 macrophages to achieve an MOI of 10 bacteria per cell. They were placed on ice for 5, 15 or  
597 30 min. At each time point the extracellular supernatant was taken for CFU determination.  
598 Cultures were then washed with PBS and the final wash again taken for CFU determination to  
599 determine no residual bacteria remained. Cultures were then treated with saponin and lysed  
600 and CFU estimated as described previously [63].

601

### 602 **FcR, CR3 or zymosan phagocytosis**

603

604 Macrophages were challenged with either IgM-iC3b or IgG-opsonised SRBC or zymosan for  
605 up to 60 min as described [36]. Briefly, for CR3-mediated phagocytosis, SRBCs were washed  
606 in PBS/BSA 0.1% and incubated for 30 min with rotation in rabbit IgM anti-SRBCs. They  
607 were washed and incubated in complement C5-deficient serum without rotation for 20 min at  
608 37°C. SRBCs were further washed, re-suspended in phagocytosis medium and added to  
609 macrophages to give approximately 10 SRBCs per cell. The plates were centrifuged at room  
610 temperature at 502 x g for 2 min and then placed at 37°C for various time points. At each time  
611 point, cells were washed with room temperature phagocytosis medium and fixed in warm 4%  
612 paraformaldehyde (Sigma-Aldrich) at room temperature for 15 min and then treated with  
613 0.05M NH<sub>4</sub>Cl/PBS1X for 10 min. For FcR-mediated phagocytosis, SRBCs were washed as  
614 above and opsonized for 30 min with rotation in rabbit IgG anti-SRBCs. They were further  
615 washed, re-suspended in phagocytosis medium and added to macrophages as above. All  
616 subsequent steps were as for CR3. For zymosan uptake, zymosan was washed twice in  
617 PBS/BSA 0.1% and then re-suspended in phagocytosis medium. Macrophages were  
618 challenged with zymosan for 60 min and all subsequent steps were as described above.

### 619 **Fluorescence Microscopy and phagocytosis quantification**

620 For FcR or CR3 mediated phagocytosis cultures were washed in 1XPBS/2% FCS and  
621 external SRBCs were labelled for 30 min with F(ab')<sub>2</sub> anti-rabbit IgG Alexa Fluor 488 in  
622 PBS/2% FCS. Cells were then washed with 1XPBS/2%FCS and re-fixed in 4% PFA for 15  
623 min at room temperature and then treated with 0.05M NH<sub>4</sub>Cl for 10 min before being  
624 permeabilized in 1XPBS/2%FCS/0.05% saponin. Intracellular SRBCs were then detected  
625 using a Cy5-labeled F(ab')<sub>2</sub> anti-rabbit IgG and F-actin was stained using phalloidin-Cy3 in  
626 1XPBS/2%FCS/0.05% saponin for 30 min. After washing in 1XPBS/2%FCS/0.05% saponin  
627 cells were stained with DAPI for 5 min and mounted using Fluormount G (Interchim). For  
628 zymosan uptake, cultures were washed in 1XPBS/2% FCS and external zymosan was  
629 detected with an anti- zymosan antibody for 30 min followed by Cy5-labeled F(ab')<sub>2</sub> anti-  
630 rabbit IgG for 30 min. Cultures were washed in 1XPBS/2% FCS and permeabilized in  
631 1XPBS/2%FCS/0.05% saponin before labelling with phalloidin Cy3 to detect F-actin. After  
632 washing as above, cultures were treated with DAPI for 5 min and mounted using Fluormount  
633 G. To quantify phagocytosis, the number of internalized SRBCs/zymosan per cell was  
634 counted in 30 cells randomly chosen on the coverslips corresponding to the phagocytic index.  
635 The index obtained was divided by the index obtained for control cells and was expressed as a  
636 percentage of control cells. To determine the index of association, the total number of bound  
637 and internalized SRBCs in a cell was divided by the total number of macrophages counted.  
638 Image acquisition was performed on an inverted wide-field microscope (Leica DMI6000)  
639 with a 100× (1.4 NA) objective and a MicroMAX camera (Princeton Instruments). Z-series of  
640 images were taken at 0.3-µm increments. Analyses were performed using custom-made  
641 ImageJ (National Institutes of Health) routines.

642

### 643 **Quantification of F-actin recruitment in phagocytic cups and in macrophages**

644

645 Quantification was performed as described previously [69]. Briefly, quantification was  
646 performed on ImageJ 64bit software (NIH libraries) on a selected region in 1 place of a 16-bit  
647 stack that was acquired. Primary fluorescence intensities through the phagocytic cup and in  
648 the cell cortex were measured and background corrected. Ratio values were calculated by  
649 dividing the fluorescence intensities in the phagocytic cups by the fluorescence intensities in  
650 the cell cortex's and plotted. To quantify the F-actin intensity in HRV16 exposed  
651 macrophages two macros were used. The first macro was written to quantify the intensity of  
652 staining in the entire field of cells. Quantification was performed using ImageJ 64bit software

653 on entire 16-bit Z stacks. The macro automatically decided which plane of the Z stack to use  
654 and when in focus it divided the field into 4 sections and quantified the fluorescence of the  
655 punctate F-actin and the total F-actin in each section. The second macro was written to  
656 quantify the intensity of the punctate F-actin per cell. This was done in the same way as for  
657 macro 1 apart from instead of calculating the intensity in the section, the macro quantified it  
658 in each individual cell within the field selected by the macro. To quantify the F-actin intensity  
659 each macro used the FindFocussedSlices filter and two automatic ImageJ thresholding. The  
660 first was Percentile Dark for the total F-actin and the second was Default Dark for the  
661 punctate F-actin. The results from each output were reported as a summary for each cell or  
662 field in ImageJ. All results for both macro 1 and 2 were transferred to Graphpad Prism and  
663 plotted.

664

#### 665 **Phagosome closure assay using Total Internal Reflection Fluorescence Microscopy** 666 **(TIRFM)**

667 IgG-RBCs were centrifuged onto 35mm glass bottom dishes (MatTek Corporation) pre-  
668 treated with 0.01% poly-L-Lysine in PBS for 30 min at RT. The dishes were then washed  
669 once with a 10% BSA in PBS and incubated for 30 min with 10% BSA in PBS. Then the  
670 dishes were incubated with pre-warmed serum-free microscopy medium. RAW264.7  
671 macrophages were resuspended and allowed to sediment onto opsonized SRBC-coated dishes  
672 at 37°C.

673 TIRFM was performed using a Till PHOTONICS iMIC microscope equipped with an oil-  
674 immersion objective (Apo N 100x, NA1.49 Olympus America Inc.), a heating chamber, and  
675 two cameras: a cooled iXonEM camera and an iXon3 897 Single Photon Detection EMCCD  
676 Camera (Andor Technology). The critical angle was verified at the beginning of each session  
677 by scanning through incident angles of 0-5° to maximise evanescent wave induced  
678 fluorescence. Excitation was performed with a 491 nm and a 561 nm laser. For the  
679 phagosome closure assay, streams of 550 frames were acquired at 50 ms per frame in TIRF  
680 mode, and 50 ms per frame in epifluorescence mode with Polychrome illumination at a 3 µm  
681 increment every 2 s. TIRFM image streams were processed using ImageJ Color Profiler  
682 software (NIH).

683

#### 684 **Migration assay**

685 hMDMs were plated in IBIDI (15 microns) microscopy slides in 8 wells, treated or not with  
686 the virus for 1 h, before washing and overnight rest in the incubator. They were then  
687 incubated with sirDNA at 0.33  $\mu$ M and observed with a 20x, 1.4 NA, PH DIC objective with  
688 a spinning disk confocal (Yokogawa CSU-X1M1) inverted microscope (Leica DMI6000)  
689 equipped with a CoolSnap HQ<sup>2</sup> camera (Photometrics) and a heated chamber with CO<sub>2</sub> in a  
690 BSL3 laboratory. Acquisition was performed with MetaMorph 7.5.5 (Molecular Devices) in  
691 the bright field (BF) and CY5 channel excited with a 635 nm laser.

692

### 693 **Western blots**

694 Macrophages were lysed with lysis buffer (20 mM Tris HCl, pH 7.5, 150 mM NaCl, 0.5 %  
695 NP-40, 50 mM NaF, and 1 mM sodium orthovanadate, supplemented with complete protease  
696 inhibitor cocktail (Roche Diagnostic)) for 15 min. Lysates were centrifuged at 16,100  $\times$  g for  
697 10 min at 4°C. The supernatants were removed and stored at -20 °C and an equal  
698 concentration of protein (BCA dosage kit, Pierce) was analyzed by SDS-PAGE. Proteins were  
699 transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore) at 4°C for 100  
700 min and incubated in blocking solution TBS/0.1% Tween-20 supplemented with 5% milk or  
701 BSA for 2 h. Blots were rinsed with TBS/0.1% Tween-20 and primary antibodies were  
702 incubated in the blocking solution overnight or for 2 h as required. The membrane was further  
703 washed and incubated with HRP-coupled secondary antibodies in blocking buffer for 45 min.  
704 Detection was performed using ECL Dura substrate (GE Healthcare) and bands imaged by  
705 Fusion (Vilber Lourmat) and quantified in ImageJ.

### 706 **Scanning electron microscopy**

707 Cells were fixed with 2.5 % glutaraldehyde (Sigma Aldrich) in 0.1M HEPES, pH 7.2 in  
708 phagocytosis medium at 37°C for 1 h. They were then fixed in 2.5% glutaraldehyde in 0.1M  
709 HEPES buffer in PBS at 4°C overnight. Post fixation was done with 1 % osmium tetroxide  
710 (Merck) and 1.5 % ferrocyanide (Sigma Aldrich) in 0.1M HEPES. After dehydration by a  
711 graded series of ethanol, the samples were transferred to a Leica EM CPD300 and dried  
712 according to standard procedures. Samples were mounted on aluminium stubs and sputter  
713 coated with 7 nm of gold palladium in a Gatan ion beam coater. Samples were examined at  
714 5kV in an Jeol 6700F scanning electron microscope.

715

### 716 **siRNA Treatment**



717 Macrophages at day 7 were washed twice with macrophage medium and kept in macrophage  
718 medium at 37°C. The siRNA solution was prepared in OptiMEM medium (GlutaMAX  
719 supplemented, Gibco), containing Lipofectamine RNAiMAX reagent (Invitrogen) and siRNA  
720 at a concentration of 100 µM. siRNA was added to each well and cultures left for 96 h at  
721 37°C before being used. siRNA sequences were as follows: CGU ACG CGG AAU ACU  
722 UCG A55 (siLuciferase\_pGL2), GGA GAA CUG AUC GAU GUA UCU 55 (siArpin.1) and  
723 GCU UCC UCA UGU CGU CCU ACA 55 (siArpin.2).

724

## 725 **qPCR**

726 hMDMs were exposed to HRV16 or MI control as described above. After overnight rest,  
727 cultures were washed with PBS and RNA was extracted as previously described [70]. Briefly,  
728 hMDMs were washed with PBS at room temperature and lysed using Trizol reagent  
729 (ThermoFischer Scientific). Proteins (organic phase) and RNA and DNA (aqueous phase)  
730 were separated using chlorophorm for 2 min at room temperature followed by 15 min  
731 centrifugation at 4°C at 12,000 x g. The aqueous phase was collected and isopropanol was  
732 added to precipitate RNA and incubated for 10 min at room temperature. Samples were  
733 centrifuged for 20 min at 4°C at 15,000 x g and the pellet of RNA washed with 75% ethanol  
734 and centrifuged for a further 5 min at 4°C at 10,000 x g. The pellet was dried at room  
735 temperature and resuspended in pure water and warmed at 55°C for 5 min. The total amount  
736 of RNA was quantified using nanodrop. For reverse transcription 1 µg of mRNA was retro-  
737 transcribed into DNA using SuperScript II Reverse Transcriptase (ThermoFischer Scientific).  
738 qPCR was performed using the LightCycler 480 SYBR Green I Master (Roche) with specific  
739 oligos to detect Arpin with 18S RNA as control (Table 2). The relative quantification of Arpin  
740 gene expression was assessed using the  $\Delta\Delta C_t$  method [71].

741

## 742 **Statistical analysis**

743 Statistical tests were performed using Graphpad prism® version 6 software. All statistical  
744 tests are listed in the figure legends and significance was determined if  $p < 0.05$ .

745

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757

### 758 **Author contributions**

759

760 Conceptualization: JJ, NK and FN

761 Methodology: JJ, FH, EI, LO, PB and FN

762 Investigation: JJ, KAG, FH, AM, PB

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764 Writing: original draft: JJ, KAG, FH and FN

765 Writing: review and editing: GM, DMC, NK, FN

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768 Funding acquisition: GM, DMC, NK and FN

769

### 770 **Competing interests**

771 NK, DMC, LO, EI and GM are employed by the commercial company AstraZeneca and  
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774

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972

973

974

975 **Figure legends**

976

977 **Figure 1**

978 **HRV16 impairs bacterial internalisation by macrophages.**

979 **A-H** hMDMs were challenged with HRV16, HRV16<sup>UV</sup> or MI and then exposed to either  
980 (A, E) NTHi, (B, F) *Moraxella catarrhalis*, (C, G) *Staphylococcus aureus*, or (D, H)  
981 *Pseudomonas aeruginosa* to assess internalisation (A-D) or binding (E-H). n=3 biological  
982 replicates on different donors \*\*p<0.01, \*\*\*\*p<0.0001 Two Way Anova with Dunnett's Post  
983 Test vs MI.

984 **I, J** (I) Non-smoker, non-COPD or (J) Healthy smoker, non-COPD AM were challenged  
985 with HRV16 or controls and then exposed to NTHi to assess internalisation. n=4 biological  
986 replicates on different donors \*\*p<0.01, \*\*\*p<0.001, \*\*\*\*p<0.0001 Two Way Anova with  
987 Dunnett's Post Test vs MI.

988 Data information: Error bars represent standard error of the mean (SEM).

989

990 **Figure 2**

991 **HRV16 impairs receptor-mediated internalisation by macrophages.**

992 **A-C** hMDMs were challenged with HRV16 or controls and then exposed to (A) zymosan  
993 for 60 min, n=3 donors, 30 random cells/coverslip, (B) IgM-iC3b-opsonised SRBC for 60  
994 min, n=5 donors, 30 random cells/coverslip, or (C) IgG-opsonised SRBC for 60 min, n=5  
995 donors, 30 random cells/coverslip. \*\*\*p<0.001, \*\*\*\*p<0.0001 One Way Anova with  
996 Bonferonni's Post Test vs MI. Error bars represent standard error of the mean (SEM).

997 (D) Representative images of MI or HRV16 challenged hMDMs at 60 min post IgM-iC3b  
998 SRBC internalisation. Intracellular particles are labeled with Cy5-coupled F(ab')<sub>2</sub> anti-rabbit  
999 Ig and DAPI. Scale bar, 15 µm.

1000 (E) Representative images of MI or HRV16 challenged hMDMs at 60 min post IgG-SRBC.  
1001 Intracellular particles are labeled with Cy5-coupled F(ab')<sub>2</sub> anti-rabbit Ig and DAPI. Scale  
1002 bar, 15 µm.

1003

1004 **Figure 3**

1005 **HRV16 impairs phagocytic cup extension.**

1006 hMDMs were challenged with HRV16 or MI and then exposed to IgG-opsonized SRBC for  
1007 2-30 min.



1008 (A) Representative images of a Z projection of phalloidin staining at 5 and 15 min post  
1009 internalisation in MI and HRV16 challenged hMDMs. Scale bar, 15  $\mu$ m.  
1010 (B) Quantification of the number of actin cups in hMDMs, n=3 donors, at least 80 cups at the  
1011 early time points and 8-80 cups at later time points depending on the time  
1012 (C) Quantification of the initial F-actin enrichment around internalized particles in hMDMs,  
1013 [on n=3 different donors.](#)  
1014 (D) Quantification of internalisation of SRBC over 30 min, n=3 donors, 30 random cells/  
1015 coverslip.  
1016 (E) Quantification of association of SRBC to hMDMs, n=3 donors, 30 random cells/coverslip.  
1017 (F) Scanning electron microscopy images after 5 min of contact with IgG-opsonised SRBC  
1018 showing differences in cup formation. Scale bar, 1  $\mu$ m.  
1019 Data information: \*\*p<0.01, \*\*\*p<0.001, \*\*\*\*p<0.0001 One Way Anova with Bonferonni's  
1020 Post Test vs MI. Error bars represent standard error of the mean (SEM).

1021

## 1022 **Figure 4**

### 1023 **HRV16 downregulates Arpin expression in macrophages.**

1024 hMDMs were challenged with HRV16 or controls and then stained or lysed.

1025 (A) Representative images of MI or HRV16 challenged hMDMs stained with phalloidin-Cy3  
1026 at 24 h post infection. Scale bar, 15 $\mu$ m.

1027 (B) Quantification of the total F-actin intensity per field, n=3 donors, 20 random fields per  
1028 coverslip

1029 (C) Quantification of the punctate F-actin intensity per field, n=3 donors, 20 random fields  
1030 par coverslip.

1031 (D) Quantification of the punctate F-actin intensity per cell in 20 cells per donor per  
1032 condition, n=3 different donors.

1033 (E) Immunoblot of lysates from hMDMs challenged with MI, HRV16<sup>UV</sup> or HRV16 to detect  
1034 Arpin or actin.

1035 (F) Quantification of immunoblots normalized to MI control, n=10 experiments [on different](#)  
1036 [donors.](#)

1037 (G) Quantification of Rac1, Cdc42, p16-Arc, and p34-Arc, n=10 experiments [on different](#)  
1038 [donors.](#)

1039 (H) Quantification of total and phosphorylated cofilin expression, n=9 experiments [on](#)  
1040 [different donors.](#)

1041 (I) Relative activation of cofilin vs total cofilin, n=9 experiments [on different donors.](#)

1042 (J) RPE-1-FcγRIIA cells challenged with mock medium (MI) and transfected with plasmids  
1043 encoding EGFP (left) or EGFP-Arpin (right).

1044 (K) Quantification of FcR phagocytosis in RPE-1-FcγRIIA cells challenged with mock  
1045 medium (MI) or HRV16 and then transfected to express EGFP or EGFP-Arpin, n=4  
1046 experiments, between 30 and 50 random cells.

1047 Data information: In (B-D) \*\*p<0.01, \*\*\*p<0.001, \*\*\*\*p<0.0001 Paired T test vs MI. In (F)  
1048 \*p<0.05 One Way Anova with Bonferonni's Post Test vs MI. In (K) \*\*p<0.01, \*\*\*\*p<0.0001  
1049 unpaired T test. Error bars represent standard error of the mean (SEM).

1050

## 1051 **Figure 5**

### 1052 **Arpin knockdown impairs bacterial internalisation by macrophages.**

1053 hMDMs were non-treated, treated with siLuciferase or 2 different Arpin siRNA sequences for  
1054 96 h.

1055 (A) Immunoblot against Arpin and tubulin in siRNA-treated hMDMs.

1056 (B) Quantification of the expression of Arpin normalized to tubulin and presented relative to  
1057 non-treated as a percentage, n=9 donors, \*\*\*\*p<0.0001 One Way Anova with Bonferonni's  
1058 Post Test vs non-treated.

1059 C-F (C) NTHi internalisation, (D) *Moraxella catarrhalis* internalisation, (E)  
1060 *Staphylococcus aureus* internalisation, and (F) *Pseudomonas aeruginosa* internalisation, n=3  
1061 donors, \*\*p<0.01, \*\*\*p<0.001, \*\*\*\*p<0.0001 Two Way Anova with Dunnett's Post Test vs  
1062 non-treated.

1063 Data information: Error bars represent standard error of the mean (SEM).

1064

## 1065 **Figure 6**

### 1066 **Arpin knockdown impairs efficient phagocytic cups formation, is recruited in** 1067 **membrane extensions and at the closure site.**

1068 hMDMs were non-treated, treated with siLuciferase or 2 different Arpin siRNA sequences for  
1069 96 h, then allowed to internalize IgG-opsonised SRBC for up to 30 min and F-actin structures  
1070 around phagocytosed particles was assessed using phalloidin-Cy3.

1071 (A) Representative images at 15 and 30 min post internalisation. Scale bar, 15µm.

1072 (B) Quantification of internalisation of SRBC over 30 min, n=3 donors, 30 random  
1073 cells/coverlip.

1074 (C) Quantification of association of SRBC to hMDMs over 30 min, n=3 donors, 30 random  
1075 cells/coverlip.

1076 (D) Quantification of the number of F-actin cups in hMDMs over 30 min, n=3 donors, at least  
1077 60 cups in the early time point and between 15-60 depending on time point.

1078 (E) Quantification of the initial F-actin enrichment around internalized particles in hMDMs  
1079 over 30 min, n=3 donors, at least 60 cups in the early time point and between 15-60  
1080 depending on time point,

1081 (F) Scanning electron microscopy images following Arpin knockdown or control and 15 min  
1082 IgG-opsonised SRBC internalisation showing differences in membrane recruitment. Scale  
1083 bar, 1  $\mu$ m.

1084 (G) Phagosome closure assay to detect the site of phagosome closure in the TIRFM mode  
1085 (left) and the base of the phagosome in the epifluorescence mode after shifting the stage 3  $\mu$ m  
1086 in z.

1087 (H) Phagosome closure assay was performed using RAW264.7 macrophages transiently  
1088 expressing both Lifeact-mCherry and EGFP-Arpin. Lifeact-mCherry (upper panels) and EGFP-  
1089 Arpin (lower panels) imaging by TIRFM and epifluorescence were performed alternatively at  
1090 37°C every 2 s for 10 min. Transmitted light images are presented at the bottom.

1091 Data information: \*p<0.05, \*\*p<0.01, \*\*\*p<0.001, \*\*\*\*p<0.0001 One Way Anova with  
1092 Bonferonni's Post Test vs MI. Error bars represent standard error of the mean (SEM).

1093

#### 1094 **Figure EV1**

1095 **HRV16 impairs NTHi internalisation by macrophages from TCID<sub>50</sub> 1 x 10<sup>3</sup>.**

1096 A-E hMDMs were challenged with HRV16, HRV16<sup>UV</sup> or MI at various doses and then  
1097 exposed to NTHi for up to 2 h. (A) TCID<sub>50</sub> 1 x 10<sup>1</sup>; (B) TCID<sub>50</sub> 1 x 10<sup>3</sup>; (C) TCID<sub>50</sub> 1 x 10<sup>5</sup>;  
1098 (D) TCID<sub>50</sub> 1 x 10<sup>6</sup>; (E) TCID<sub>50</sub> 3 x 10<sup>7</sup>. n=3 donors \*\* p<0.01, \*\*\*\*p<0.0001 Two Way  
1099 Anova with Dunnett's Post Test vs MI.

1100 (F) Quantification of the percentage inhibition in NTHi uptake vs MI at 120 min in HRV16  
1101 exposed hMDMs, n=3 donors, \*\*\*\*p<0.0001 One Way Anova with Dunnett's Post Test vs  
1102 TCID<sub>50</sub> 1 x 10<sup>1</sup>.

1103 Data information: Error bars represent standard error of the mean (SEM).

1104

#### 1105 **Figure EV2**

1106 **HRV16 impairs *Staphylococcus aureus* internalisation by macrophages from TCID<sub>50</sub> 1 x  
1107 10<sup>3</sup>.**

1108 A-E hMDMs were challenged with HRV16, HRV16<sup>UV</sup> or MI at various doses and then  
1109 exposed to *Staphylococcus aureus* for up to 2 h. (A) TCID<sub>50</sub> 1 x 10<sup>1</sup>; (B) TCID<sub>50</sub> 1 x 10<sup>3</sup>; (C)

1110 TCID<sub>50</sub> 1 x 10<sup>5</sup> ; (D) TCID<sub>50</sub> 1 x 10<sup>6</sup> ; (E) TCID<sub>50</sub> 3 x 10<sup>7</sup>, n=3 donors \*p<0.05,  
1111 \*\*\*\*p<0.0001 Two Way Anova with Dunnett's Post Test vs MI.

1112 (F) Quantification of the percentage inhibition in *Staphylococcus aureus* uptake vs MI at 120  
1113 min in HRV16 exposed hMDMs, n=3 donors, \*\*\*\*p<0.0001 One Way Anova with Dunnett's  
1114 Post Test vs TCID<sub>50</sub> 1 x 10<sup>1</sup>.

1115 Data information: Error bars represent standard error of the mean (SEM).

1116

### 1117 **Figure EV3**

1118 **Arpin knockdown does not impair bacterial binding to hMDMs.**

1119 **A-D** hMDMs were non-treated, treated with siLuciferase or 2 different Arpin siRNA  
1120 sequences for 96 h and bacterial binding at 4°C assessed over 30 min. (A) NTHi ; (B)  
1121 *Moraxella catarrhalis* ; (C) *Staphylococcus aureus* ; (D) *Pseudomonas aeruginosa*, n=3  
1122 donors. Error bars represent standard error of the mean (SEM).

1123

### 1124 **Figure EV4**

1125 **HRV16 or Arpin knockdown does not cause a delay in internalisation in hMDMs.**

1126 **A,B** hMDMs were challenged with HRV16, HRV16<sup>UV</sup> or MI, or non-treated, treated with  
1127 siLuciferase or 2 different Arpin siRNA sequences for 96 h and internalisation of IgG-  
1128 opsonised SRBC assessed over 60 min. (A) HRV16 exposure, n=5 donors. (B) Arpin  
1129 knockdown, n=3 donors, \*\*\*\*p<0.0001 One Way Anova with Bonferonni's Post Test vs non-  
1130 treated. Error bars represent standard error of the mean (SEM).

1131

### 1132 **Figure EV5**

1133 **FcgRIIA-expressing RPE-1 cells phagocytose IgG-opsonized SRBC.**

1134 (A) RPE-1 or RPE-1-FcgRIIA cells were labeled with an anti-CD32 antibody or an isotype  
1135 control antibody coupled to PE. Fluorescence was analyzed by flow cytometry (BD Accuri™  
1136 C6, 10 000 cells per condition).

1137 **B-D** (B) Phagocytosis of IgG-opsonized or non-opsonized SRBC was assessed in RPE-1 or  
1138 RPE-1 FcgRIIA cells, and quantified for (C) SRBC association at 0 min (between 13 and 16  
1139 cells). Scale bar, 30 μm. One representative experiment was quantified. \*\*\*\*p<0.0001,  
1140 Unpaired T test, and (D) internalization at 60 min (between 10 and 12 cells) \*\*\*p=0.0002,  
1141 Unpaired T test. Error bars represent standard error of the mean (SEM).

1142

1143

1144 **Table 1**

1145 **Patient demographics for AM samples used in figure 1**

	Non-smoker	Healthy smoker
Male/female	3/1	3/1
Age	44 (average)	46 (average)
Ex/current smoker	N/A	0/4

1146

1147 **Table 2: List of qPCR primers used in this study**

Gene	Sequence	Reference
<b>18S histone</b>	<b>Forward:</b> <b>AGGAATTGACGGAAGGGCAC</b> <b>Reverse:</b> <b>GGACATCTAAGGGCATCACA</b>	<b>Génin <i>et al.</i>, 2015 Plos Pathogens 11: e1004971</b>
<b>Arpin</b>	<b>Forward:</b> <b>CTTCCTCATGTCGTCCTACAAGGT</b> <b>G</b> <b>Reverse:</b> <b>CTGTCAGCGCGAGCAGCTCT</b>	<b>Dang <i>et al.</i>, 2013 Nature Letters 503: 281</b>

1148

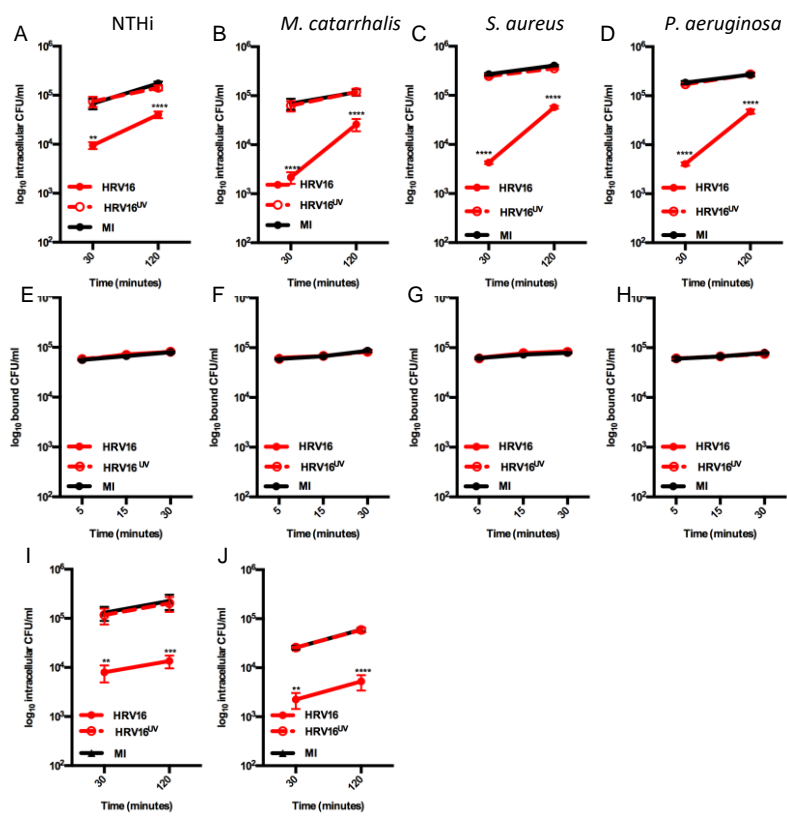


Figure 1

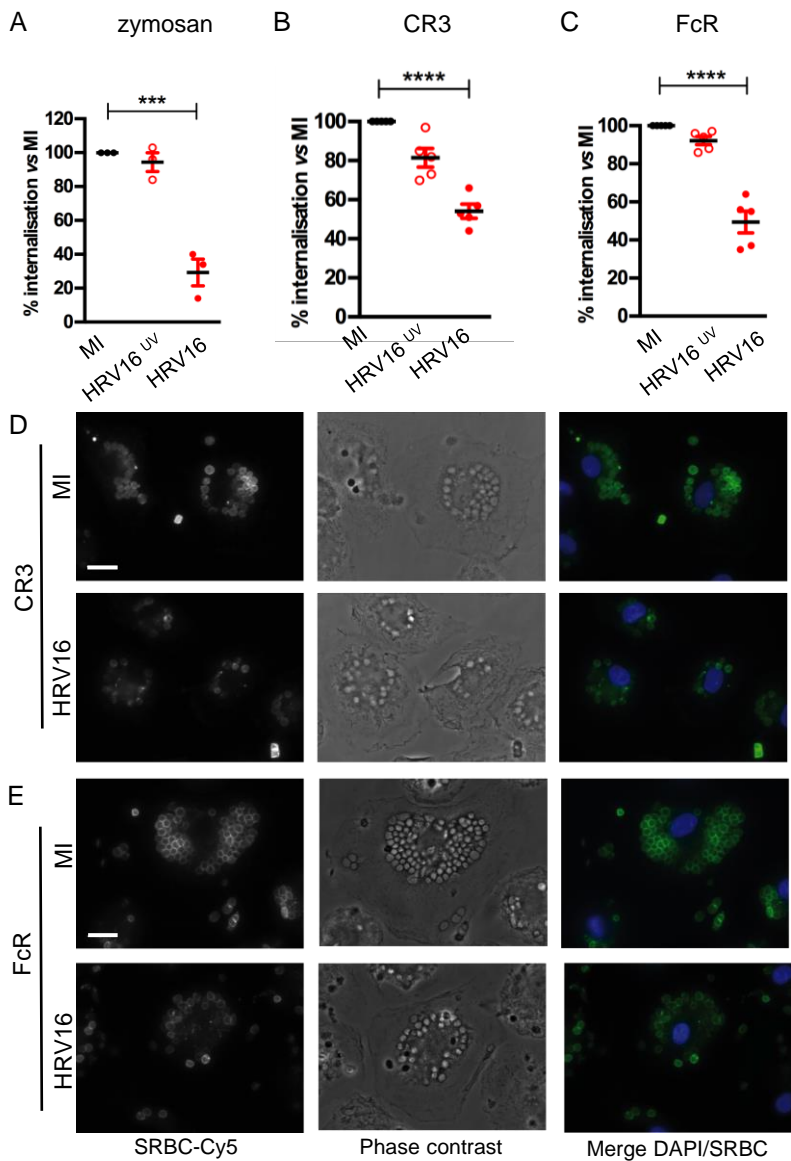


Figure 2

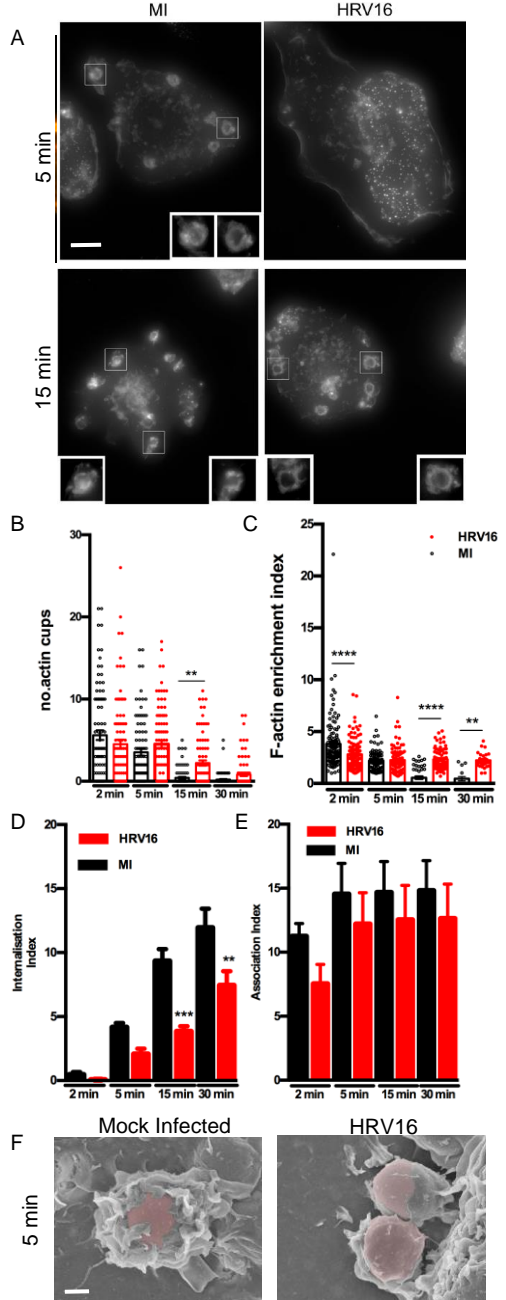


Figure 3



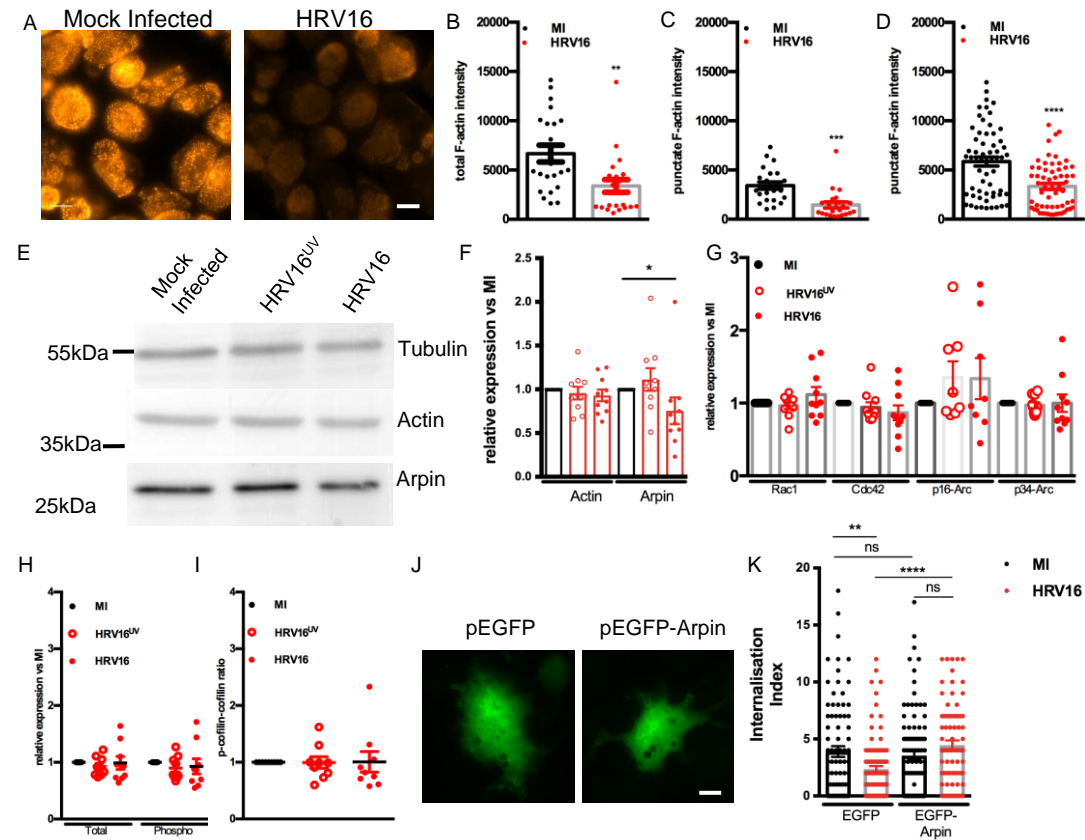


Figure 4

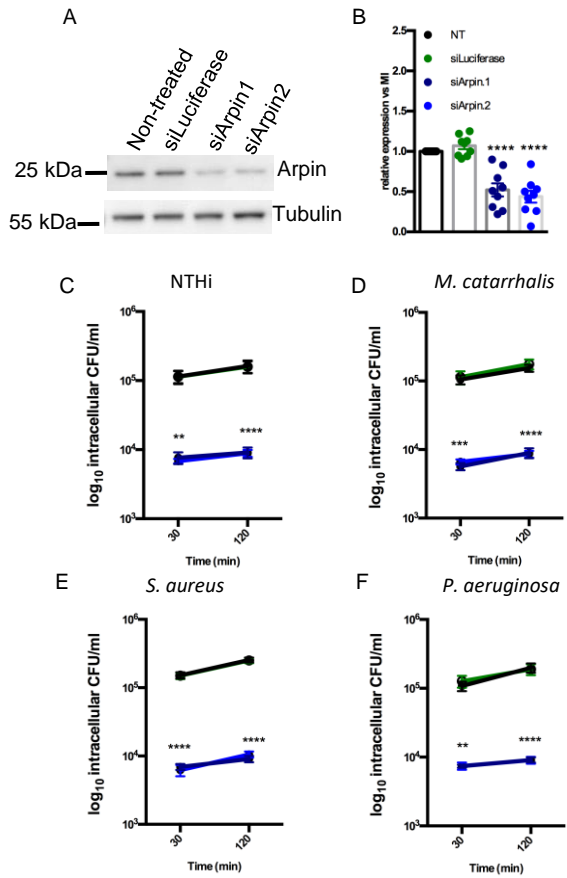


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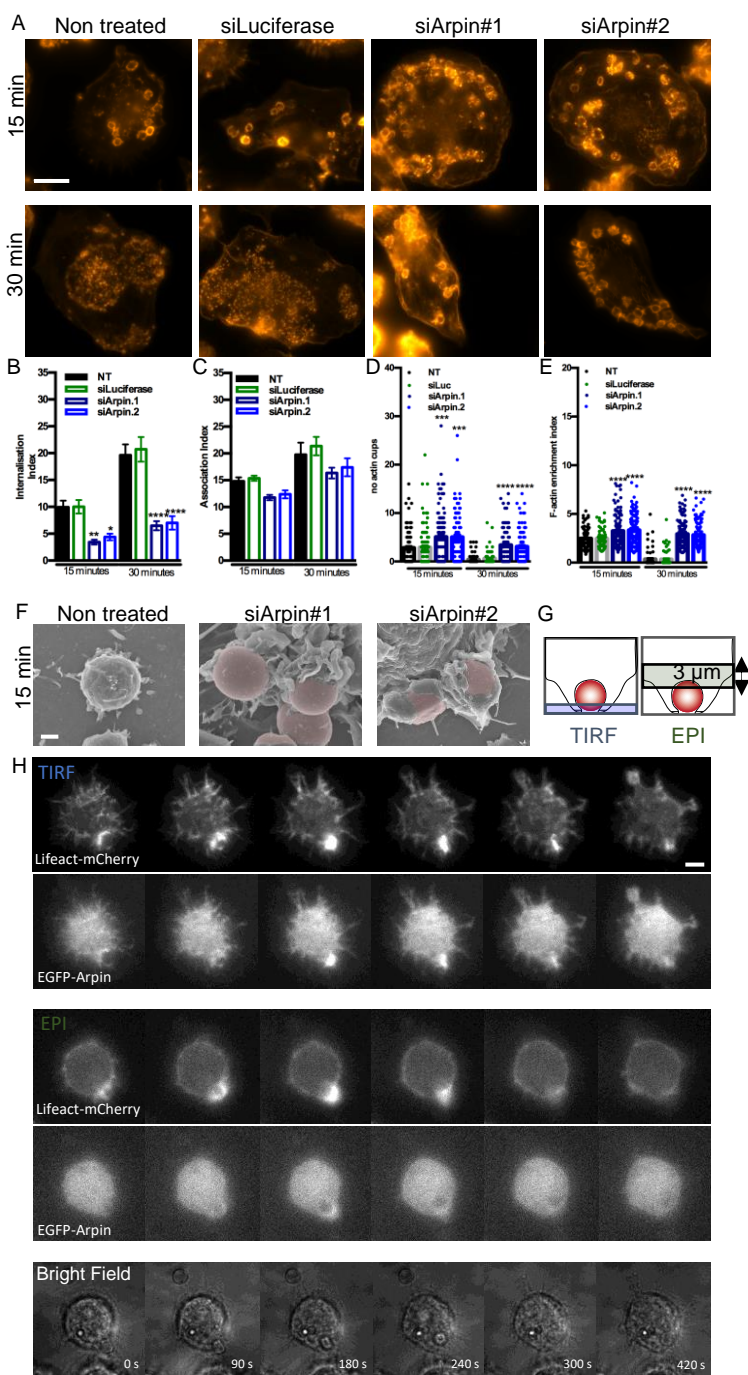


Figure 6

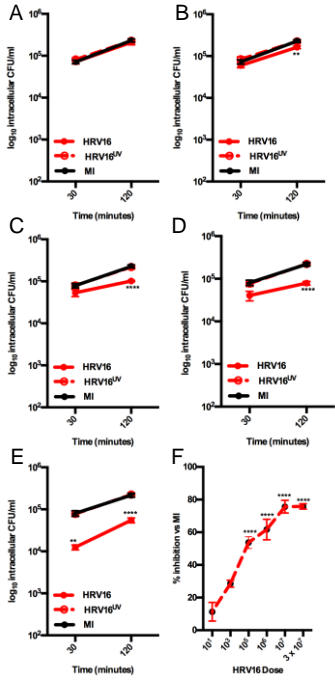


Figure EV1

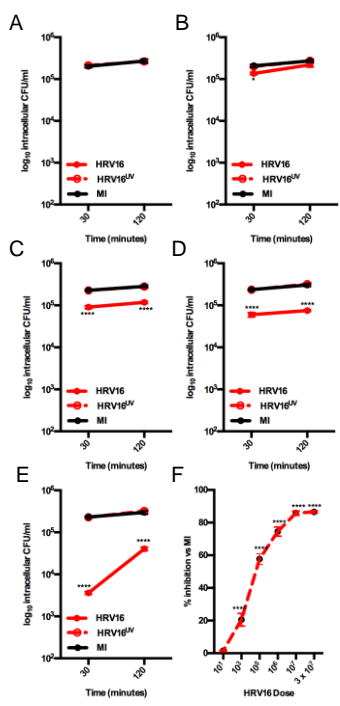


Figure EV2

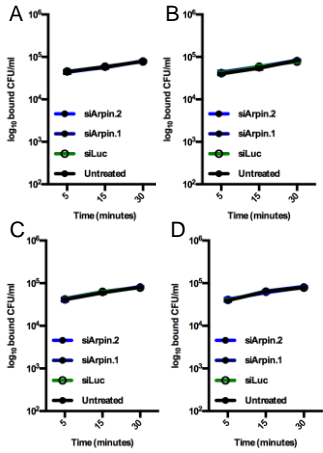


Figure EV3

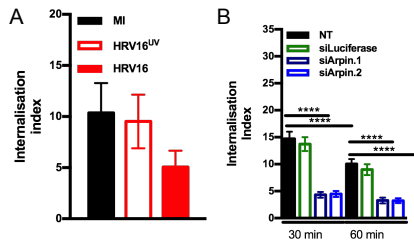


Figure EV4

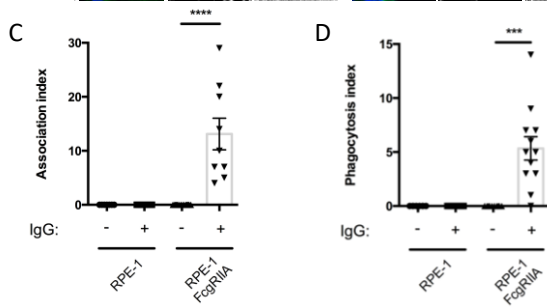
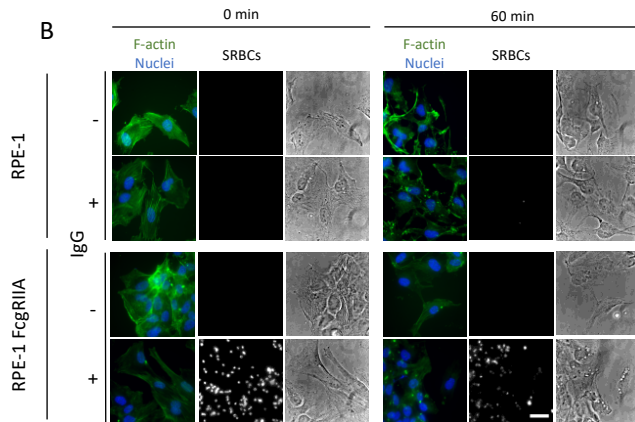
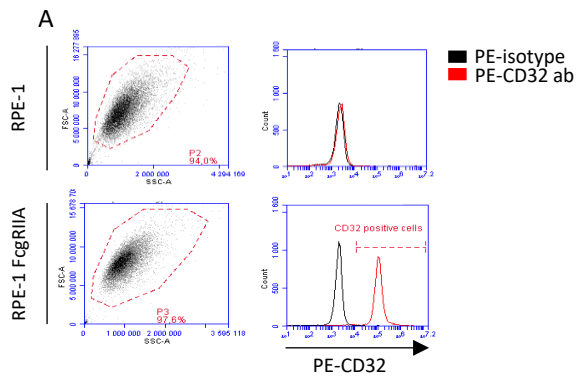


Figure EV5